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Novel function of serine protease HTRA1 in inhibiting adipogenic differentiation of human mesenchymal stem cells via MAP kinase-mediated MMP upregulation

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ABSTRACT

Adipogenesis is the process by which mesenchymal stem cells (MSCs) develop into lipid-laden adipocytes. Being the dominant cell type within adipose tissue, adipocytes play a central role in regulating circulating fatty acid levels, which is considered to be of critical importance in maintaining insulin sensitivity. High temperature requirement protease A1 (HTRA1) is a newly recognized regulator of MSC differentiation, although its role as a mediator of adipogenesis has not yet been defined. The aim of this work was therefore to evaluate HTRA1's influence on human MSC (hMSC) adipogenesis and to establish a potential mode of action. We report that the addition of exogenous HTRA1 to human hMSCs undergoing adipogenesis suppressed their ability to develop into lipid laden adipocytes. These effects were demonstrated as being reliant on both its protease and PDZ domain, and were mediated through the actions of c-Jun N-terminal kinase (JNK) and matrix metalloproteinases (MMPs). The relevance of such findings with regards to HTRA1's potential influence on adipocyte function *in vivo*, is made evident by the fact that HTRA1 and MMP-13 were readily identifiable within crown-like structures present in visceral adipose tissue samples from insulin resistant obese human subjects. These data therefore implicate HTRA1 as a negative regulator of MSC adipogenesis and are suggestive of its potential involvement in adipose tissue remodeling under pathological conditions.

INTRODUCTION

Multipotent mesenchymal stem cells (MSCs) represent an important source of adipocytes, undergoing adipogenesis in two phases consisting of commitment to pre-adipocytes and their terminal differentiation into mature adipocytes [1, 2]. Of critical importance in the body's ability to adapt to energy requirements, is the capacity for pre-adipocytes to develop into adipocytes and for them to be able to acquire more fat. Both these conditions require alterations in cell shape and volume, and are heavily reliant on the versatility and integrity of the adipocyte extracellular matrix (ECM), termed basal lamina [3]. The basal lamina is composed of numerous core proteins, including type IV collagen [4, 5], various laminin isoforms [5], and heparan sulfate proteoglycan (HSPG) [6] to name but a few, and is subject to continual turnover mediated by a wide variety of matrix metalloproteinases (MMPs) [7, 8]. Interference with basal lamina dynamics may therefore have significant consequences to adipogenesis and subsequent adipocyte stability.

Dysregulation of adipogenesis and adipose tissue remodeling has been linked to several human disorders, most notably of which is obesity-induced insulin resistance [9, 10]. A characteristic feature of obese adipose tissue is the presence of crown-like structures consisting mainly of infiltrating macrophages surrounding dead adipocytes [11]. Exposure to such an environment not only impairs adipocyte responsiveness to insulin, but can also lead to deviations in the differentiation status of pre-adipocytes [9]. As such, there is currently a strong focus on the identification of key factors involved in the regulation of adipogenic commitment.

High temperature requirement protease A1 (HTRA1) has been newly identified as a modulator of human MSC (hMSC) differentiation, whereby it acts to enhance osteoblast

formation most likely through proteolytic modification of the ECM, and its expression levels in bone tissue coincide with the appearance of new bone formation during fracture repair in mice [12]. Furthermore, preliminary findings from loss of function studies have suggested that HTRA1's ability to influence hMSC lineage commitment may extend to adipogenic differentiation [12]. However, the extent to which these effects are mediated by secreted HTRA1 and the possible mechanisms underlining its mode of action remain to be determined. Certainly, the growing number of reports pertaining to HTRA1's ability to modulate the ECM both under normal physiological conditions and in disease, may provide some basis for how it could potentially interact with and regulate adipocyte formation [13-15].

To define HTRA1's role in adipogenesis and its potential physiological relevance, we investigated the effects of exogenously added recombinant HTRA1 on the development of hMSCs into mature adipocytes and further analysed its expression levels in visceral adipose tissue from obese patients. We demonstrate that HTRA1 acts to significantly suppress adipocyte development, primarily through upregulation of JNK activity and MMP production. We further localized HTRA1 and MMP-13 to sites of crown-like structures within the adipose tissue of an insulin resistant obese patient. This work thus identifies HTRA1 as being a potentially novel mediator of adipose tissue homeostasis.

MATERIALS AND METHODS

Materials

Anti-ERK1/2, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-SAPK/JNK, anti-phospho-SAPK/JNK (Thr183/Tyr185), anti-p38, anti-phospho-p38 (Thr180/Tyr182) were all purchased from Cell signaling Technology (Leiden, The Netherlands). Anti-tubulin was from Sigma-Aldrich. Anti-type IV collagen (M3F7) and anti-laminin γ-1 (2E8) were from the Developmental Studies Hybridoma Bank (University of Iowa). Anti-fibronectin (MAB1936) was from Merck Millipore (Schaffhausen, Switzerland). Monoclonal anti-human HTRA1 was generously supplied by Prof. Michael Ehrmann (University Duisburg-Essen, Germany) and generated as previously described [16]. Rabbit anti-MMP-13 was purchased from Abcam (Cambridge, UK). Mouse IgG, HRP- or Cy3-labeled secondary antibodies specific for mouse or rabbit IgG were purchased from Jackson ImmunoResearch (Suffolk, UK), and biotin-labeled goat anti-mouse IgG and Vectastain ABC system used in immunohistochemical staining studies was from Reactolab SA (Servion, Switzerland). Biotinylated swine anti-rabbit IgG was from Dako (Baar, Switzerland). Histidine-labeled human HTRA1 proteins were overexpressed in *E. coli* and purified using previously described methodologies [16].

hMSC Culture

The hMSCs employed in this work were provided by the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine at Scott & White through a grant from NCRR of the NIH, Grant # P40RR017447. hMSCs were isolated from bone

marrow aspirates and multipotency fully defined in accordance with the minimal criteria outlined by International Society for Cellular Therapy [17]. hMSCs were maintained at 37°C, in 5% CO_2 and 98% humidity in normal growth medium consisting of Dulbecco's modified eagle medium (DMEM-low glucose, with GlutaMAX) (Life Technologies, Zug, Switzerland), supplemented with 10% fetal bovine serum (FBS, BioWest), penicillin/streptomycin (50 units/ml; 50 μg/ml), and used between passage 5 and 8 unless otherwise stated.

Adipogenic differentiation of hMSCs

The adipogenic differentiation of hMSCs was performed using a protocol previously established in our laboratory [12]. Briefly, hMSCs seeded in cell culture plates at 10'000 cells/cm^2 were incubated for 3 days with adipogenic induction medium consisting of normal growth medium (DMEM-high glucose, GlutaMAX) supplemented with 1 μM dexamethasone, 10 μg/ml insulin, 0.1 mM Indomethacin, and 0.5 mM isobutylmethylxanthine (IBMX) (all from Sigma-Aldrich). Cells were subsequently cultured in adipogenic maintenance medium consisting of IBMX-free adipogenic medium, and replenished with fresh medium every 72 h for up to 14-24 days unless otherwise stated. Adipocyte formation was confirmed by positive staining of lipid droplets by Oil Red O (Sigma-Aldrich). Oil Red O staining was quantified by extraction with isopropanol absorption measured at 510 nm using a Multiplate reader (Infinite M200, Tecan). Values were normalized to cell number assessed by DAPI staining as described below. For recombinant HTRA1 addition studies, hMSCs undergoing adipogenesis were treated with recombinant HTRA1 starting at day 3 after adipogenic induction. Dose response

experiments determined 45 nM to be the most appropriate concentration of HTRA1 to use for these studies (Supporting Information Fig. S1A). For inhibition studies, cells were treated with recombinant HTRA1 in the presence of MMP-3 inhibitor NNGH, MMP-13 inhibitor CL-82198, or MAP Kinase inhibitors PD98059, SP600125 and SB239063 (all from Enzo Life Science, Lausen, Switzerland). DMSO (0.05%) was added as vehicle control and was equivalent to highest concentration of inhibitor used.

RT-qPCR

Gene expression levels were quantified by reverse-transcription quantitative PCR (RT-qPCR) using TaqMan Gene Expression Assays (Life Technologies) (Supporting Information Table S1) as previously described [12]. Total RNA was harvested from cells at selected time points during differentiation and 0.5µg of total RNA reverse-transcribed using Superscript II (Life Technologies). An equivalent of 10ng total RNA was applied as cDNA template in the successive qRT-PCR reaction using the StepOnePlus (Life Technologies). Values were normalized to *GUSB* mRNA levels and presented as fold change as compared to control cells or cells treated with vehicle alone (value = 1) according to the $2^{-\Delta\Delta CT}$ method.

siRNA Assays

Silencing of *HTRA1* and *MMP13* gene expression was performed with Silencer Select siRNA oligos (Life Technologies) according to the manufacturer's protocol as previously described [12]. For *HTRA1* knockdown, hMSCs (1 x10^5) were transfected with 40 nM *HTRA1*-specific (s11279, s11280) or negative control siRNA (Negative Control-1) using

the NEON Transfection System (Life Technologies). Transfected cells were immediately seeded in cell culture plates with fresh growth medium (without antibiotics) and incubated for 24 h at 37°C, 5% CO2. Medium was then replaced with either fresh growth medium or adipogenic differentiation and total RNA and supernatants harvested at selected time points for further analysis. For *MMP13* knockdown, adherent hMSCs were initially seeded in cell culture plates and cultured in induction medium for 3 days. Thereafter, hMSCs were transfected for 24 h with 40 nM *MMP13*-specific (11496, 104024) or negative control siRNA (Negative Control-1) using Lipofectamine RNAiMAX (Life Technologies). Supernatants were subsequently replaced with adipogenic maintenance medium and total RNA and supernatants harvested at selected time points for further analysis. The effect of *HTRA1* and *MMP13* gene silencing on lipid droplet formation was evaluated by Oil Red O staining as described above.

Proteolysis Assays

HTRA1 mediated digestion of BODIPY-FL-labeled DQ-elastin or DQ-type IV collagen was performed using EnzChek assay kits (Life Technologies) as previously described [18].

ELISAs

Extracellular protein levels of HTRA1, MMP-3, MMP-13, syndecan-1 and -4 were measured in the supernatants of hMSCs at selected time points by ELISA. The HTRA1 ELISA was performed as previously described [19]. The MMP-3, MMP-13, Syndecan-1 and -4 specific ELISAs were performed using Duo-Set ELISA Development Systems according to the manufacturer's instructions (R & D Systems, Abingdon, UK).

FACS Analysis

Subconfluent adherent hMSCs were detached using Accutase (Life Technologies) and resuspended in ice cold FACS buffer (PBS/BSA 0.1%) in round bottom 96-well plates to 2 x10^6/ml. Cells were incubated on ice for 1 h with equimolar concentrations of his-labeled recombinant HTRA1 either alone, or in combination with heparin, heparanase, chondroitinase (all from Sigma-Aldrich), collagenase (Roche), or peptide competitors of integrin binding CS1 and GRGDSP (from ANAWA Trading SA, Zurich, Switzerland). Cells were then washed in cold FACS buffer and incubated with a FITC conjugated rabbit anti-6xHis tag antibody (Lucerna-Chem, Luzern, Switzerland) on ice for 1 h. Bound HTRA1 was detected by FACS analysis using a FACS-Canto II (BD Biosciences) and quantified using FlowJo 10 software.

Protein Electrophoresis and Immunoblotting

Whole cell extracts from untreated or HTRA1 treated hMSCs were harvested using CelLytic M (Sigma Aldrich) supplemented with protease and phosphatase inhibitor cocktails (Sigma Aldrich) and 1mM PMSF at 2 weeks post adipogenic induction. Protein concentrations were determined by Bradford-based protein assay (Bio-Rad). Protein samples were boiled for 5 min in loading buffer (50 mM Tris-HCl, pH 6.8, 2% (v/v) SDS, 10% (v/v) glycerol, 100 mM DTT, 0.002% (w/v) bromophenol blue) and equal amounts of protein analyzed by SDS-PAGE using or 10% or 4-15% precast Tris-HCl gels (BioRad) under reducing conditions and electroblotted onto PVDF membranes using the Trans-Blot Turbo blotting system (BioRad). Membranes were then blocked with 5% (w/v) skim milk

in TBST (50mM Tris-HCl, pH 7.6, 150mM NaCl, 0.05% (v/v) Tween 20) for 1 h at room temperature and then incubated with specific antibodies overnight at 4°C at recommended dilutions in blocking buffer. Antibody binding was detected using HRP-conjugated secondary antibodies followed by incubation in Super Signal West Pico or West Dura Chemiluminescent Substrate (Life Technologies) and exposed to x-ray film.

Preparation and identification of Fnfs

For the identification of native Fnfs in supernatants, hMSCs undergoing adipogenic differentiation for 14 days were treated for a further 24 h with HTRA1 (45 nM) in fresh growth medium without FCS. Where indicated, HTRA1 was also added in combination with CL-82198 (20 nM). Supernatants were then harvested and immediately placed on ice. Cellular debris was removed by centrifugation and the remaining supernatant concentrated 40-fold by centrifugation at 3000G for 30 min in Amicon Ultra columns (10 kDa size exclusion, Millipore). Concentrated protein solutions were then supplemented with protease inhibitor cocktail (1:100, Sigma Aldrich) and 1mM PMSF and separated on a 4-15% SDS-PAGE pre-cast gel (BioRad). Methods for preparing purified Fnfs generated from HTRA1-digested plasma fibronectin were carried out as previously described [18]. Fnfs were visualized by immunoblotting using a specific antibody against the N-terminus of fibronectin and detected using an HRP-conjugated secondary antibody as described above.

VLDL uptake

Lipid uptake by hMSCs undergoing adipogenesis was performed using DiI-labeled human VLDL (KALEN Biomedical) at late time points of adipogenesis between days 18-24. Cultured hMSCs were rinsed once with PBS and then incubated for 3 h under standard culture conditions in assay buffer supplemented with DiI-labeled VLDL (4 μg/ml) and carrier protein ApoE2 (3 μg/ml, PeproTech, UK). Following incubation, cells were washed 3 times with PBS and fixed with PBS-buffered formaldehyde (4%) for 30 min. Nuclei were stained with DAPI (1:10'000) in PBS for 10 min and mounted in Mowiol containing 2.5% (w/v) DABCO. Images were captured using a Leica DMI6000B automated inverted research microscope system (Leica Microsystems). Relative mean fluorescence intensity (RMFI) was quantified using NIH ImageJ software as described previously with some modifications [20]. Briefly, images were processed using identical image acquisition settings and exposure times. TIFF files were converted to 8-bit gray-scale mode and inverted onto a white background. Negative and positive staining controls were used to set measurable limits and threshold levels, and were then applied to all samples. Finally, RMFI was calculated as mean grey value per area and normalized to the number of cells per image as assessed by automated counting of DAPI-positive nuclei staining using the ImageJ software.

Immunofluorescence staining

hMSCs undergoing adipogenesis were fixed with PBS-buffered formaldehyde (4%) for 30 min, washed 3 times with PBS and then blocked for 30 min in 2% BSA and 5% normal goat serum in PBS. Cells were incubated overnight at 4^{o}C with a monoclonal mouse anti-type IV collagen or monoclonal mouse anti-Laminin γ-1 antibodies, or an isotype control

at an equivalent concentration. Antibody binding was detected using a Cy3-conjugated secondary antibody and nuclei stained with DAPI. Cells were mounted with Mowiol containing 2.5% DABCO and images captured using a Leica DMI6000B automated inverted research microscope system (Leica Microsystems). Positive staining was quantified as described above. RMFI was quantified using NIH ImageJ software as described above.

Immunohistochemical staining

Dewaxed paraffin sections of adipose tissue were rehydrated and blocked in normal serum (Jackson ImmunoResearch) for 30 min. Sections were then incubated for 1 h at 37^{o}C with monoclonal mouse anti-HTRA1 (2.5 μg/ml) or rabbit anti-MMP-13 (5 μg/ml) and staining specificity controlled for using either mouse or rabbit IgG at equivalent concentrations. Sections were then washed in PBS and incubated with biotinylated goat anti-mouse IgG (1:200) or swine anti-rabbit IgG (1:400) for 1 h at 37^{o}C followed by washing and a further incubation for 30 min with Vectastain. Sections were then developed using 3,3' diaminobenzidine tetrahydrochloride (DAB), counterstained with Harris' Hematoxylin and mounted in Mowiol.

Human Patients

Visceral tissue was obtained during bariatric surgery from morbidly obese, non-diabetic patients (Supporting Information Table S2). All adipose tissue donors gave written informed consent before the study, which had been approved by the Ethics Committee of the Medical Faculty, University of Leipzig. Insulin sensitivity was defined by the glucose

infusion rate (GIR) during the steady state of an euglycemic hyperinsulinemic clamp as previously described [21].

Statistical Analysis

Two-tailed unpaired Student's *t*-test for comparison of two groups or one-way analysis of variance (ANOVA) with Tukey's post hoc test for multiple group comparisons were used. In all cases, a *P*-value of < 0.05 was considered statistically significant, and all data were expressed as mean ± standard deviation (S.D).

RESULTS

HTRA1 suppresses lipid uptake and droplet formation in hMSCs undergoing adipogenesis

Preliminary studies utilizing an HTRA1-specific ELISA were conducted to investigate HTRA1 production by hMSCs. In undifferentiated control hMSCs, secreted HTRA1 levels increased over culture time, reaching a maximum level of 9.6 ± 0.5 ng/ml after 10 days of culture (Fig. 1A). HTRA1 production also increased over time in hMSC cultures undergoing adipogenesis, although levels were significantly reduced as compared to the undifferentiated control hMSCs throughout the 2 week culture period. Interestingly, these observations are in direct contrast to the stimulatory effects of osteogenic induction on HTRA1 production by hMSCs (Supporting Information Fig. S1B) [12]. These data therefore suggest that reductions in HTRA1 might be involved in adipogenic differentiation of hMSCs. To address this model, we effectively eliminated endogenous

HTRA1 from the culture system through the use of small interfering RNA (siRNA) (Supporting Information Fig. S1C) and assessed the ability of hMSCs to undergo adipogenesis using Oil Red O staining. Indeed, hMSCs deficient in HTRA1 produced significantly more lipid droplets than those treated with control siRNA, thereby indicating that loss of HTRA1 had an overall positive influence on adipocyte formation (Fig. 1B). Furthermore, stimulation of HTRA1 deficient hMSCs with functional HTRA1 could reduce Oil Red O staining to a level comparable to that observed in control cultures (Fig. 1C). These studies also provided the first evidence that the addition of exogenous HTRA1 acts to suppress lipid droplet formation in developing adipocytes. Note that an HTRA1 variant (ΔMac) was used that is lacking its N-terminal Mac domain, for which no function is known to date [22] (Supporting Information Fig. S1D). Furthermore, N-terminal truncation does not affect HTRA1 function (Supporting Information Fig. S1E) and therefore this variant is termed HTRA1 throughout.

As HTRA1 contains both a C-terminal PDZ domain and a functional serine protease domain, we asked whether either domain was required for determining HTRA1's inhibitory influence over hMSC adipogenesis. Therefore, we generated several different proteolytically active and inactive recombinant HTRA1 proteins (Supporting Information Fig. S1D and S1E) and assessed their ability to influence oil droplet formation in hMSCs undergoing adipogenesis. Indeed, inactivation of HTRA1's proteolytic activity through replacement of residue Ser328 with Ala (HTRA1 S328A), significantly impaired it's ability to inhibit lipid droplet formation in hMSCs undergoing adipogenesis (Fig. 1D). Similarly, deletion of the PDZ domain in proteolytically active HTRA1 (HTRA1ΔPDZ) also abolished its inhibitory action on hMSC adipogenesis. Molecular based analyses

revealed HTRA1 treatment had no significant influence on the expression levels of adipogenic-associated markers *PPARG*, *FABP4* and *CD36,* and only a minimal, but significant inhibitory effect on the expression level of the adipocyte-specific marker *ADIPOQ* (1.3-fold; $p < 0.01$) (Supporting Information Fig. S2). Therefore, HTRA1's capacity to regulate adipogenic gene expression was not considered to be sufficient to account for its inhibitory actions on oil droplet formation in hMSCs undergoing adipogenic differentiation. When considering alternative routes through which HTRA1 could modulate hMSC adipogenesis, we became aware of a previous study in which HTRA1 was shown to cleave Xenopus syndecan-4 [23]. Syndecans are cell surface HSPGs and represent a predominant feature of developing adipocytes, in which they play a central role in mediating lipid uptake [6]. In order to investigate this, we assessed the potential for various recombinant HTRA1 proteins to bind to hMSCs using fluorescence activated cell sorting (FACS) analysis. We could demonstrate that intact, but proteolytically inactive HTRA1 S328A was able to bind to hMSCs in a PDZ-dependent manner (Fig. 2A). The involvement of the PDZ domain in cellular binding of HTRA1 was further substantiated through the use of a recombinant HTRA1 containing the PDZ domain only (HTRA1Δprotease) (Fig. 2B). We next investigated whether removal of heparan sulfate (HS) using heparanase or competition with heparin could reduce cellular binding of HTRA1, and thereby provide support for an HTRA1-HSPG interaction. Indeed, the ability of HTRA1 S328A (Fig. 2C) and HTRA1Δprotease (Fig. 2D) to bind to hMSCs was markedly reduced by pre-treatment with heparanase. Similarly, pre-treatment of cells with heparin could also dramatically reduce cellular binding of both HTRA1 S328A (Fig. 2E) and HTRA1Δprotease (Fig. 2F). By contrast, replacement of heparanase with collagenase

(Supporting Information Fig. S3A) or chondroitinase (Supporting Information Fig. S3B), and heparin with integrin-binding competitor peptides CS-1 (Supporting Information Fig. S3C) or GRGDSP (Supporting Information Fig. S3D), had little or no effect on the cellular binding capacity of HTRA1's PDZ domain.

Although these data demonstrated HTRA1's ability to bind to HSPG, they fell short of confirming whether HSPG was a proteolytic substrate of HTRA1. To address this question, we used an ELISA to determine levels of cleaved soluble syndecan-4 in supernatants from hMSCs at various stages of adipogenesis. Low levels of soluble syndecan-4 were observed at day 10 (4.2 pg/ml ± 1.5) and day 21 (16.4 pg/ml ± 1.9) in hMSCs undergoing adipogenesis (Fig. 2G). By contrast, significantly higher soluble syndecan-4 levels were measured in hMSCs treated with HTRA1 at day 10 (44.8 pg/ml ± 3.5; $P < 0.001$) and day 21 (84 pg/ml ± 7.7; $P < 0.001$). Again, these effects were determined as being dependent on HTRA1 possessing an intact PDZ domain, and were therefore in accordance with the findings from our binding studies. Studies were also undertaken to examine soluble syndecan-1. However, protein levels remained below detection limits in all treatment groups (data not shown). Based on syndecan-4 being a prominent regulator of lipid uptake by differentiating adipocytes [6], we surmised that HTRA1-induced syndecan-4 shedding would result in significant impairment of this process. Studies to investigate active lipid uptake in hMSCs undergoing adipogenesis were therefore undertaken using fluorescently labeled VLDL (VLDL-DiI). The uptake and accrual of high levels of VLDL-DiI in normally differentiating cells was apparent after a 3 h incubation period (Fig. 2H). However, VLDL-DiI levels were significantly diminished in hMSCs that had previously been treated with HTRA1. Furthermore, the inability of

HTRA1 to exert a significant influence over VLDL-DiI uptake in the absence of either a protease or PDZ domain, convincingly supports a causal relationship between HTRA1's ability to impair lipid accrual in hMSCs undergoing adipogenesis and its propensity to interact with and cleave HSPG from the cell surface.

Induction of MMPs by HTRA1

Although HTRA1 has the potential to directly interact with and cleave HSPG, it is also possible that the observed effects are indirect e.g. via other proteases. Our previous studies utilizing synovial fibroblasts and intervertebral disc cells have identified MMPs to be strongly upregulated in response to HTRA1 stimulation, being mediated through the production of reactive fibronectin species [18, 19]. In addition to their ability to induce HSPG cleavage and shedding [24], MMPs are also potent regulators of adipogenesis, having both inhibitory and stimulatory effects [7, 8, 25]. This led us to investigate the possibility that MMP production by hMSCs undergoing adipogenesis might be regulated by HTRA1. Indeed, treatment of hMSCs undergoing adipogenesis with HTRA1 resulted in significant increases in the mRNA expression levels of *MMP1* (Fig. 3A), *MMP2* (Fig. 3B), *MMP3* (Fig. 3C), *MMP9* (Fig. 3D), and *MMP13* (Fig. 3E), although no significant alterations were observed in *MMP14* expression levels (Fig. 3F). Additionally, ELISA measurements of supernatants revealed significant increases in MMP-3 (Fig. 3G) and MMP-13 (Fig. 3H) protein levels. Small, but significant increases in *MMP9* and *MMP13* mRNA expression, along with MMP-3 and -13 protein production, were also observed in hMSCs treated with HTRA1 S328A, although to a significantly lesser degree as compared to HTRA1. In order to ascertain whether the observed increases in MMP production were

associated with increases in MMP activity, we undertook immunofluorescence studies to assess the levels of major MMP substrates present within cultures of differentiating hMSCs. The basal lamina of hMSCs undergoing adipogenesis stained positive for both laminin (Fig. 4A) and type IV collagen (Fig. 4B) at day 22 after adipogenic induction. Based on HTRA1's ability to upregulate MMP production, we additionally examined whether it could also induce alterations in type IV collagen and laminin in hMSC cultures undergoing adipogenesis. Indeed, treatment with HTRA1 resulted in a significant reduction in the levels of both laminin (Fig. 4A) and type IV collagen (Fig. 4B). Furthermore, the effects of HTRA1 were significantly more pronounced than either HTRA1 S328A or HTRA1ΔPDZ, and were therefore in accordance with HTRA1's ability to regulate MMP production. Although it's possible that changes in the basal lamina were additionally due to factors other than MMPs, we are confident that they were not the direct result of HTRA1's actions based on the findings from previous studies in which HTRA1 failed to degrade either type IV collagen or laminin [13]. Indeed, we were only able to observe partial degradation of type IV collagen by HTRA1 at concentrations starting from 0.86 μM, 20-fold higher than that used in our culture system (Supporting Information Fig. S4).

As HTRA1-generated fibronectin fragments (Fnfs) are known to be potent stimulators of MMP production [18, 19], we additionally investigated whether such fragments could be identified within hMSCs undergoing adipogenesis. Protein analysis of supernatants from hMSCs identified several Fnf species, including the well described 29 kDa Fnf (Supporting Information Fig. S5A) [18, 19]. Interestingly, levels of this particular fragment decreased upon adipogenic induction of control cultures, but was still present in

the supernatants of HTRA1-treated hMSC cultures undergoing adipogenesis. Furthermore, proteolytic inactive HTRA1 S328A failed to generate elevated levels of Fnfs, thereby confirming it to be dependent on HTRA1's protease activity. In order to investigate whether the 29 kDa Fnf had the same capabilities as HTRA1 in terms of stimulating MMP production by hMSCs undergoing adipogenesis, we purified HTRA1-generated Fnfs (Supporting Information Fig. S5B) and assessed their ability to influence MMP expression in comparison to HTRA1. Although Fnfs at a final concentration of up to 40 μg/ml were able to stimulate *MMP1* (Supporting Information Fig. S5C), *MMP3* (Supporting Information Fig. S5D) and *MMP13* (Supporting Information Fig. S5E) expression in hMSCs undergoing adipogenesis, levels were significantly reduced as compared to HTRA1 treatment. Moreover, the addition of Fnfs failed to have any significant impact on the level of oil droplet formation in hMSCs undergoing adipogenesis (Supporting Information Fig. S5F). It therefore seems unlikely than Fnfs are the major instigators of MMP production and adipogenic suppression in hMSCs in response to HTRA1 addition.

The inhibitory effects of HTRA1 are dependent on MMP and MAP kinase activation

Having established MMP overproduction as being a predominant feature in HTRA1 stimulated hMSCs, we sought to determine whether they had any significant relevance in defining HTRA1's influence over hMSC adipogenesis. In order to investigate this, we utilized MMP inhibitors NNGH and CL-82198 at concentrations deemed to be selective for the inhibition of MMP-3 and MMP-13 respectively [26, 27], as well as siRNA targeted knockdown of *MMP13* gene expression. Initial immunofluorescence studies confirmed that type IV collagen levels were significantly elevated in HTRA1-treated cultures when

exposed to NNGH (Fig. 5A) or CL-82198 (Fig. 5B) as compared to hMSCs treated with HTRA1 alone. Similarly, both NNGH (Fig. 5C) and CL-82198 (Fig. 5D) significantly reduced HTRA1's ability to generate cleaved soluble syndecan-4 in hMSC cultures undergoing adipogenesis in a concentration dependent manner. Based on our assumption that alterations in basal lamina composition are the predominant driving force behind HTRA1's anti-adipogenic effects, we anticipated that the inhibition of either MMP-3 or MMP-13 in HTRA1-treated cultures should preserve adipocyte development as compared to hMSCs treated with HTRA1 alone. In order to investigate this, we quantified Oil Red O staining in HTRA1-treated hMSCs undergoing adipogenesis cultured in the presence or absence of either MMP inhibitor. Our findings confirmed that inhibition of either MMP-3 (Fig. 5E) or MMP-13 (Fig. 5F) could significantly restore oil droplet formation in HTRA1-treated hMSCs undergoing adipogenesis to levels similar to those observed in differentiating hMSCs cultured in the absence of HTRA1. These observations were additionally supported by studies in which *MMP13* gene expression was silenced in hMSCs undergoing adipogenesis. *MMP13* expression could be effectively suppressed in both HTRA1-treated and untreated hMSCs undergoing adipogenesis (Supporting Information Fig. S6A), and was shown to significantly impair HTRA1's ability to inhibit oil droplet formation as determined by quantitative analysis of Oil Red O staining (Supporting Information Fig. S6B). Furthermore, when analyzing the supernatants of HTRA1-treated hMSCs undergoing adipogenesis in which the MMP-13 inhibitor had been added, we observed a clear decrease in the amount of the HTRA1-generated 29 kDa Fnf (Supporting Information Fig. S6C and S6D). This finding therefore lends additional support to the theory that in this culture system at least, HTRA1's capacity to induce MMP

expression and impair adipogenesis in hMSCs is primarily mediated through mechanisms unrelated to Fnfs, and that at least in the case of MMP-13, Fnf generation in cultured hMSCs is actually dependent on the actions of MMPs rather than HTRA1 proteolysis directly.

Having therefore confirmed the central importance of MMPs in mediating HTRA1's effects on hMSC adipogenesis, we explored potential mechanisms through which HTRA1 could induce MMP production. Our previous findings from studies utilizing primary cultures of IVD cells have hinted towards the MAP kinase signaling pathway as playing a significant role in mediating the upregulation of MMPs in response to HTRA1 [18]. Moreover, alterations in MAP kinase activation are known to have a profound influence on both adipogenesis and adipose tissue function [28]. Based on this, we investigated the role of MAP kinase signaling in adipogenesis and evaluated its involvement in mediating HTRA1's effects on hMSC adipogenesis. Initial Western blot analysis of hMSCs undergoing adipogenesis revealed minimal levels of activated ERK, JNK and p38 at day 14 post induction (Fig. 6A). By contrast, phosphorylated levels of both ERK, JNK and p38 were noticeably increased in HTRA1-treated hMSCs in both a protease and PDZ dependent manner. In order to further investigate the involvement of MAP kinase signaling in MMP expression in hMSCs undergoing adipogenesis, we utilized MAP kinase inhibitors PD98059, SP600125 and SB239063 at concentrations deemed to be selective for the inhibition of ERK, JNK and p38 respectively [29, 30]. Treatment of hMSCs undergoing adipogenesis with MAP kinase inhibitors significantly reduced the basal expression of *MMP3* at day 17 post induction (Fig. 6B). The enhanced *MMP3* expression observed in HTRA1-treated hMSCs undergoing adipogenesis was similarly reduced

following the addition of MAP kinase inhibitors. By contrast, no significant changes in *MMP13* expression levels were observed in hMSCs undergoing adipogenesis treated with PD98059, and HTRA1's ability to upregulate *MMP13* expression was not significantly affected (Fig. 6C). However, inhibition of JNK or p38 activity had a profound effect on both basal *MMP13* levels, as well as on HTRA1-mediated upregulation of *MMP13* expression. In order to assess the possible consequence of such changes in MMP expression on hMSC adipogenesis, we investigated the effects of MAP kinase inhibitors on oil droplet formation in hMSCs undergoing adipogenesis. Neither PD98059 or SP600125 had any significant effect on oil droplet formation in normally differentiating hMSCs (Fig. 6D). By contrast, basal levels of oil droplet formation were dramatically reduced in control cultures treated with SB239063. The addition of either PD98059 or SP600125 to HTRA1-treated hMSCs undergoing adipogenesis resulted in significant increases in Oil Red O staining, and in the case of SP600125, fully restored oil droplet formation to levels comparable to those observed in cultures undergoing adipogenesis in the absence of HTRA1. However, the addition of SB239063 failed to alleviate the inhibitory effects of HTRA1, and instead led to a significant reduction in Oil Red O staining as compared to hMSCs treated with HTRA1 alone.

Detection of HTRA1 in visceral fat from obese patients

In order to validate the relevance of these findings with regards to HTRA1's potential role in mediating adipogenesis *in vivo*, immunohistochemical analysis was performed on visceral adipose tissue taken from obese patients (Supporting Information Table S2) that were diagnosed as being either insulin sensitive (IS) or insulin resistant (IR) as described

previously in detail [21]. Analysis of visceral fat from either IS (Supporting Information Fig. S7A) or IR (Supporting Information Fig. S7B) obese patients revealed HTRA1 primarily localized to large blood vessels found throughout the tissue. HTRA1 was also identified in areas of crown-like structures in both IS (Fig. 7A) and IR (Fig. 7B) (Supporting Information Fig. S8) obese patients although the level of staining at these sites was markedly enhanced in IR adipose tissue in accordance with increases in cellular content. Furthermore, HTRA1 positive crown-like structures also showed positive staining for MMP-13 (Fig. 7C), thus re-enforcing the concept of there being a close working relationship between these two proteases. In all cases, specificity of immunostaining was confirmed using relevant IgG isotype controls (Fig. 7D-F).

DISCUSSION

In the current study, we have identified HTRA1 as a potent inducer of MAP kinase-dependent MMP production in hMSC cultures undergoing adipogenesis, the result of which leads to modulation of ECM components and impaired lipid droplet accrual in differentiating cells. Furthermore, the observation that HTRA1 is present at high levels in visceral adipose tissue in crown-like structures is suggestive of its active involvement in mediating adipose tissue function under pathological conditions.

HTRA1 is now well established as a key regulator of ECM turnover, having been linked to various diseases in which the breakdown of normal ECM is a prominent pathological feature [13-16, 18]. Findings from our own studies into the role of HTRA1 in joint and IVD degeneration have led to the suggestion that HTRA1 may instigate tissue breakdown through an upregulation in MMP production, most likely via the action of

reactive Fnf species [18, 19]. In the context of adipose tissue, MMP-mediated ECM remodelling and reorganization forms an integral part of normal tissue homeostasis, being required for adipocyte differentiation and function [7, 8]. Abnormal fluctuations in MMP levels within adipose tissue may therefore represent an important factor in determining adipocyte health and subsequent risk of disease. This is supported by our current findings where increased production of MMPs by hMSCs undergoing adipogenesis in response to HTRA1 led to a loss of vital ECM components and impaired lipid accrual. Such observations are therefore suggestive of HTRA1 as having a detrimental role in hMSC adipogenesis and that these effects are related to the actions of MMPs, in particular MMP-3 and MMP-13. However, it should be noted that all studies in the current report were performed using two dimensional culture systems, and may have therefore overlooked the potential involvement of other MMPs, such as MMP-14, whose regulatory role in adipogenesis is only made apparent when examined under three dimensional culture systems [31]. Furthermore, in contrast to previous studies [18, 19], the effects of HTRA1 do not appear to be reliant on the generation of Fnfs. In fact, we provide evidence to suggest that MMP-13 itself may be responsible for the appearance of such fragments in response to HTRA1. As such, alternative mechanisms must exist through which HTRA1 can instigate changes in hMSC MMP production and adipogenic potential.

Some insights into HTRA1's mode of action could be gleaned through manipulation of its trypsin-like serine protease domain and its protein-binding PDZ domain. Certainly, both domains were of critical importance in determining HTRA1's inhibitory influence over hMSC adipogenesis, being required for both efficient MMP production as well as for the observed decreases in type IV collagen, laminin and lipid

uptake in hMSCs undergoing adipogenesis. Of particular interest was the novel finding that HTRA1 could interact with surface bound HS in a PDZ-dependent manner. It is possible therefore that sequestration of HTRA1 to the cell surface by HS would allow for close interactions to form between HTRA1 and HS-containing glycoproteins. This concept is supported by the finding that increased levels of soluble syndecan-4 could be detected in in the supernatants of differentiating hMSC cultures treated with structurally intact HTRA1. Syndecans are important regulators of lipid uptake in differentiating adipocytes [6, 32] and their liberation from the cell surface by proteolytic cleavage may constitute a potential cause of diminished lipid accrual in hMSCs undergoing adipogenesis following HTRA1 treatment. In addition to syndecans, numerous other glycoproteins also exist within the adipocyte ECM, including the previously identified HTRA1 substrate nidogen [13]. Nidogen represents an important stabilizing component of the adipocyte basal lamina through its binding to type IV collagen and laminin [33]. The possible targeting of nidogen by HTRA1 may therefore represent an alternative means through which the ECM could be affected by HTRA1, and adipogenesis inhibited. Interestingly, nidogen cleavage by HTRA1 also has the potential to generate various fragment species *in vitro*, although their influence on MMP production and adipogenesis remains to be determined.

HTRA1's ability to both induce MMP production and inhibit hMSC adipogenesis was additionally identified as being dependent on the activities of JNK, and to a lesser extent, ERK. Interpretation of the data concerning the involvement of p38 were hampered by the fact that inhibition of p38 activation resulted in a significant reduction in oil droplet formation in hMSCs undergoing adipogenesis regardless of treatment conditions. The regulation of MMP expression by MAP kinases is well documented [reviewed in 34], and

several studies have now identified ERK and JNK as potent negative regulators of MSC adipogenesis [35-37]. Despite having demonstrated the importance of MAP kinase activation in mediating the effects of HTRA1 during hMSC adipogenesis, we were unable to ascertain the cause for such signaling events. Certainly, both the proteolytic activity and PDZ domain of HTRA1 were essential requirements for efficient MAP kinase activation, thereby inferring substrate binding and degradation were necessary. One potential means by which HTRA1 could invoke MAP kinase activation in the context of the current study, is through syndecan-4 shedding, whereby disruption of the syndecan-4 to MAP kinase signaling cascade may have contributed to increases in JNK activity levels [38].

The relevance of such findings is made apparent by the fact that both MAP kinases and MMPs are centrally linked to the pathophysiology of adipose tissue, being implicated in the development of insulin resistance in obese mice and humans [28, 39, 40]. Adipocyte dysfunction clearly plays a major role in determining the severity of insulin resistance among obese patients and as such, factors which negatively influence adipocyte development are likely to contribute to and potentially exacerbate the disease state [9]. In this regard, inappropriately high levels of HTRA1 in the adipose tissue of obese individuals may be viewed as being potentially harmful and an indication of deficiencies in adipocyte development and function. The significance of this is made evident by the fact that HTRA1 could be detected in the visceral fat from a small cohort of obese patients, and that levels were greatest at sites where crown-like structures were most apparent. This, together with the fact that MMP-13 was also localized to these same regions, lends further support to the concept of HTRA1 representing a novel mediator of adipogenesis and fat turnover.

CONCLUSIONS

In conclusion, our findings identify high levels of exogenous HTRA1 as having a negative influence of hMSC adipogenesis *in vitro*, being mediated through activation of MAP kinase signaling and subsequent increases in MMP production. Furthermore, elevated levels of HTRA1 and MMP-13 proteins were evident in crown-like structures in visceral adipose tissue samples of insulin-resistant obese patients. Such observations may therefore be of relevance when considering HTRA1's potential role in the underlying processes governing adipose tissue and adipocyte dysregulation.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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Figure 1. HTRA1 suppresses lipid uptake and droplet formation in hMSCs undergoing adipogenesis. **(A):** hMSCs were incubated in growth medium (control) or adipogenic induction medium (adipogenic) for up to 14 days and secreted HTRA1 protein levels determined by ELISA. *$p < 0.001$ as compared to hMSCs undergoing adipogenesis. **(B):** hMSCs were treated with scrambled siRNA (scr) or siRNAs specific for HTRA1 (H1 and H2) and induced to undergo adipogenesis. On day 14 following adipogenic induction, cells were stained using Oil Red O and oil droplet accrual quantified and normalized to cell number. Scale bar = 1 mm. **(C):** hMSCs were treated with siRNA specific for HTRA1 (H2), HTRA1 (45 nM) or a combination of both H2 and HTRA1 and induced to undergo adipogenesis. On day 14 following adipogenic induction, cells were stained using Oil Red O and oil droplet accrual quantified and normalized to cell number. *$p < 0.01$ as compared to siRNA (scr) treated cells. **(D):** hMSCs were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1), proteolytic inactive HTRA1 (HTRA1 S328A) or proteolytic active HTRA1 without PDZ domain (HTRA1ΔPDZ). On day 14 following adipogenic induction, cells were stained using Oil Red O and oil droplet accrual quantified and normalized to cell number. *$p < 0.001$ as compared to untreated. Scale bar = 1 mm. Data are representative of at least 2 separate experiments performed in triplicate. Abbreviations: hMSC, human mesenchymal stem cells; ELISA, enzyme-linked immunosorbent assay; HTRA1, high temperature requirement protease A1; siRNA, small interfering RNA.

Figure 2. HTRA1 interacts with surface bound HSPG. **(A-F):** hMSC were incubated with recombinant his-labeled HTRA1 protein (0.45 μM) on ice for 1 h, washed and HTRA1

binding determined by FACS analysis using a FITC labeled anti-histidine antibody. hMSC binding of PDZ domain-containing HTRA1 S328A **(A)** or HTRA1Δprotease **(B)** as compared to HTRA1ΔPDZ. In order to investigate the involvement of HSPG in the binding of recombinant HTRA1 protein, cells were pre-treated with heparanase (10 U/ml) or heparin (10 μg/ml). hMSC binding of S328A **(C, E)** and HTRA1Δprotease **(D, F)** in the presence of heparanase **(C, D)** or heparin **(E, F)**. **(G):** hMSCs were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1), proteolytic inactive HTRA1 (HTRA1 S328A) or proteolytic active HTRA1 without PDZ domain (HTRA1ΔPDZ). ELISA measurements of soluble syndecan-4 were performed at day 10 and 21 post adipogenic induction. **(H):** hMSCs were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1), proteolytic inactive HTRA1 (HTRA1 S328A) or proteolytic active HTRA1 without PDZ domain (HTRA1ΔPDZ). At day 24 post adipogenic induction, hMSCs were rinsed once with PBS and incubated for an additional 3 h with DiI-labeled VLDL and carrier protein ApoE2. VLDL-DiI (red) uptake was quantified by measuring relative linear median fluorescence intensity (RMFI) using NIH ImageJ software and normalized to cell number. Nuclei were stained with DAPI (blue). Scale bar = 75 μm. *$p <$ 0.001 as compared to untreated hMSCs. Data are representative of at least 2 separate experiments performed in triplicate. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1; HSPG, heparan sulfate proteoglycan; ELISA, enzyme-linked immunosorbent assay; FITC, Fluorescein isothiocyanate; VLDL-DiI, very low density lipoprotein-dialkylcarbocyanine.

Figure 3. HTRA1 upregulates MMP production by hMSCs undergoing adipogenesis. hMSCs were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1), proteolytic inactive HTRA1 (HTRA1 S328A) or proteolytic active HTRA1 without PDZ domain (HTRA1ΔPDZ). At 21 days post adipogenic induction, expression levels of *MMP1* **(A)**, *MMP2* **(B),** *MMP3* **(C)**, *MMP9* **(D)**, *MMP13* **(E)** and *MMP14* **(F)** were determined by RT-qPCR. Data was normalized to *GUSB* and expressed as fold change as compared to untreated hMSCs (value = 1) using the comparative C_T method. **(G, H):** hMSCs were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1), proteolytic inactive HTRA1 (HTRA1 S328A) or proteolytic active HTRA1 without PDZ domain (HTRA1ΔPDZ). At 21 days post adipogenic induction, levels of secreted MMP-3 **(G)** and -13 **(H)** were determined in hMSC supernatants using specific ELISAs. *$p < 0.05$, **$p < 0.001$ as compared to untreated hMSCs. Data are representative of at least 2 separate experiments performed in triplicate. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1; MMP, matrix metalloproteinase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

Figure 4. Immunofluorescence analysis of ECM. hMSCs were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1), proteolytic inactive HTRA1 (HTRA1 S328A) or proteolytic active HTRA1 without PDZ domain (HTRA1ΔPDZ). At day 24 post adipogenic induction, hMSCs were stained with antibodies specific for laminin **(A)** or type IV collagen **(B)** and

positive staining detected using a Cy3-labelled secondary antibody (red). Immunofluorescence was quantified by measuring relative linear median fluorescence intensity (RMFI) using NIH ImageJ software and normalized to cell number. Nuclei were stained with DAPI (blue). Scale bar = 100 μm. *$p < 0.01$ as compared to untreated hMSCs. Data is representative of at least 2 separate experiments performed in triplicate. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1.

Figure 5. HTRA1's effects are mediated through MMP activities. **(A, B):** hMSCs pretreated with either vehicle control or with 10 μM of NNGH **(A)** or 20 μM of CL-82198 **(B)** were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1). At day 22 post adipogenic induction, hMSCs were stained with an antibody against type IV collagen and positive staining detected using a Cy3-labelled secondary antibody. Immunofluorescence was quantified by measuring relative linear median fluorescence intensity (RMFI) using NIH ImageJ software and normalized to cell number. **(C, D):** hMSCs pretreated with either vehicle control or with varying concentrations of NNGH **(C)** or CL-82198 **(D)** were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1). At day 17 post adipogenic induction, soluble syndecan-4 levels were measured in supernatants by specific ELISA. **(E, F)**: hMSCs pretreated with either vehicle control or with varying concentrations of NNGH **(E)** or CL-82198 **(F)** were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1). At day 18 post adipogenic induction, cells were stained using Oil

Red O and oil droplet accrual quantified and normalized to cell number. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared to controls from untreated or HTRA1-treated groups respectively. Data are representative of at least 2 separate experiments performed in triplicate. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1; MMP, matrix metalloproteinase; ELISA, enzyme-linked immunosorbent assay.

Figure 6. HTRA1's effects are mediated through MAP kinase activities. **(A):** hMSCs were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1), proteolytic inactive HTRA1 (HTRA1 S328A) or proteolytic active HTRA1 without PDZ domain (HTRA1ΔPDZ). At day 14 post adipogenic induction, protein lysates were subjected to Western blot analysis using antibodies specific for phosphorylated and non-phosphorylated JNK, p38 and ERK. An anti-tubulin monoclonal was used to confirm equal loading. **(B-D):** hMSCs pretreated with either vehicle control or with PD98059 (10 μM), SP600125 (20 μM) or SB239063 (10 μM) were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1). At day 17 post adipogenic induction, expression levels of *MMP3* **(B)** and *MMP13* **(C)** were determined by RT-qPCR. Data was normalized to *GUSB* and expressed as fold change relative to untreated, control hMSCs (value = 1) using the comparative C_T method. **(D)**: Oil Red O staining was also performed and oil droplet accrual quantified and normalized to cell number. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared to controls from untreated or HTRA1-treated groups respectively. Data are representative of at least 2 separate experiments performed in

triplicate. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1; MMP, matrix metalloproteinase; MAP, mitogen-activated protein; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GUSB, Glucuronidase beta.

Figure 7. Identification of HTRA1 in adipose tissue. Paraffin wax sections of human visceral (omental) adipose tissue from insulin sensitive (IS) or insulin resistant (IR) obese patients were incubated with an anti-HTRA1 antibody **(A, B)**, anti-MMP-13 antibody **(C)** or relevant IgG controls **(D-F)**, and positive staining identified using an appropriate HRP-labeled polyclonal antibody with subsequent development using 3,3'-diaminobenzidine (brown). *Arrow heads*, HTRA1 or MMP-13 present in crown-like structures. Scale bar = 200 μm. Abbreviations: HTRA1, high temperature requirement protease A1; MMP, matrix metalloproteinase; HRP, horseradish peroxidase.

Supplementary Fig. 1. (A): hMSCs were induced to undergo adipogenesis in the presence of varying concentrations of recombinant wild type HTRA1 (HTRA1) ranging from 0 to 45 nM, and its influence on oil droplet accrual determined by Oil Red O staining. *p < 0.01 as compared hMSCs cultured in the absence of HTRA1. **(B):** hMSCs were incubated in growth medium (control), osteogenic induction medium (osteogenic) or adipogenic induction medium (adipogenic) for up to 14 days and endogenous HTRA1 protein levels measured in supernatants by specific ELISA. *p < 0.001 as compared hMSCs cultured in growth medium at the equivalent time points. **(C):** hMSCs were treated with scrambled siRNA (scr) or siRNAs specific for HTRA1 (H1 and H2) and induced to undergo adipogenesis. Endogenous levels of HTRA1 were measured in supernatants at day 3 post adipogenic induction using a specific ELISA. *p < 0.001 as compared to scrambled siRNA (scr) treated cells. **(D):** Schematic of recombinant human HTRA1 proteins; active HTRA1 (ΔMac), inactive HTRA1 (HTRA1 S328A), active HTRA1 without PDZ domain (HTRA1ΔPDZ), inactive HTRA1 without PDZ domain (HTRA1 S328AΔPDZ) and HTRA1 without protease domain (HTRA1Δprotease). The purified recombinant histidine-labeled human HTRA1 proteins were also visualized on a Coomassie blue-stained SDS-PAGE gel. *M*, protein marker; *lane 1*, HTRA1; *lane 2*, HTRA1 S328A; *lane 3*, HTRA1ΔPDZ; *lane 4*, HTRA1 S328AΔPDZ; *lane 5*, HTRA1Δprotease. **(E):** Proteolytic activity of recombinant HTRA1 proteins (45 nM) towards BODIPY-FL-labeled DQ elastin (25 μg/ml) at 37^{o}C as determined using a Multiplate reader. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1; ELISA, enzyme-linked immunosorbent assay; siRNA, small interfering RNA; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Supplementary Fig. 2. Influence of HTRA1 on adipogenic markers. hMSCs were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1), proteolytic inactive HTRA1 (HTRA1 S328A) or proteolytic active HTRA1 without PDZ domain (HTRA1ΔPDZ). At 21 days post adipogenic induction, expression levels of *PPARG* **(A)**, *FABP4* **(B)**, *CD36* **(C)** and *ADIPOQ* **(D)** were determined by RT-qPCR. Data was normalized to *GUSB* and expressed as fold change as compared to untreated hMSCs (value = 1) using the comparative C_T method. *$p < 0.01$ as compared to untreated cells. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1; PPARG, peroxisome proliferator-activated receptor gamma; FABP4, fatty acid binding protein 4; CD36, cluster of differentiation 36; ADIPOQ, adiponectin; GUSB, Glucuronidase beta; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Supplementary Fig. 3. FACS analysis of recombinant HTRA1 binding to non-differentiated hMSCs. HTRA1Δprotease (0.45 μM) was incubated with hMSCs for 1 h at 4^{o}C and cell-bound HTRA1 protein detected using a FITC labeled anti-his antibody. The influence of pre-incubating cells with collagenase (1 mg/ml) **(A)**, chondroitinase (10 U/ml) **(B)**, CS1 (50 μM) (**C**) or GRGDSP (50 μM) **(D)** on the percentage of cell-bound HTRA1Δprotease was calculated using FlowJo 10 software. Data are representative of least two individual experiments performed in duplicate. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1; FACS, fluorescence activated cells sorting.

Supplementary Fig. 4. Capacity for HTRA1 to degrade type IV collagen. Varying concentrations of HTRA1 or HTRA1 S328A were incubated with DQ-type IV collagen (25 μg/ml) at 37^{o}C and the relative fluorescence units (RFU) generated after 24 h determined using a Multiplate reader. Abbreviations: HTRA1, high temperature requirement protease A1.

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or with CL-82198 (20 μM), HTRA1 (45 nM) or HTRA1 and CL-82198. Cell supernatants were harvested, concentrated and subjected to Western blot analysis using an antibody specific for the amino-terminal fibrin- and heparin-binding domain of fibronectin. *p < 0.05, **p < 0.01, ***p < 0.001 as compared to siRNA (scr) controls from untreated or HTRA1-treated groups respectively. Data are representative of least two individual experiments performed in triplicate. *Arrow heads* indicate the 29 kDa Fnf due to HTRA1 treatment. **(D):** Coomassie stained gel of total protein from supernatants to control for equal loading. Data are representative of least two individual experiments. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GUSB, Glucuronidase beta; MMP, matrix metalloproteinase; siRNA, small interfering RNA; Fnf, fibronectin fragments.

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Supplementary Fig. 8. High power image of HTRA1 in human visceral (omental) adipose tissue from obese patient 4 (IR). Paraffin sections were incubated with an anti-HTRA1 antibody and positive staining identified using an appropriate HRP-labeled polyclonal antibody with subsequent development using 3,3'-diaminobenzidine (*brown*). Scale bar = 20 μm. Abbreviations: HTRA1, high temperature requirement protease A1; IR, insulin resistant; HRP, horseradish peroxidase.

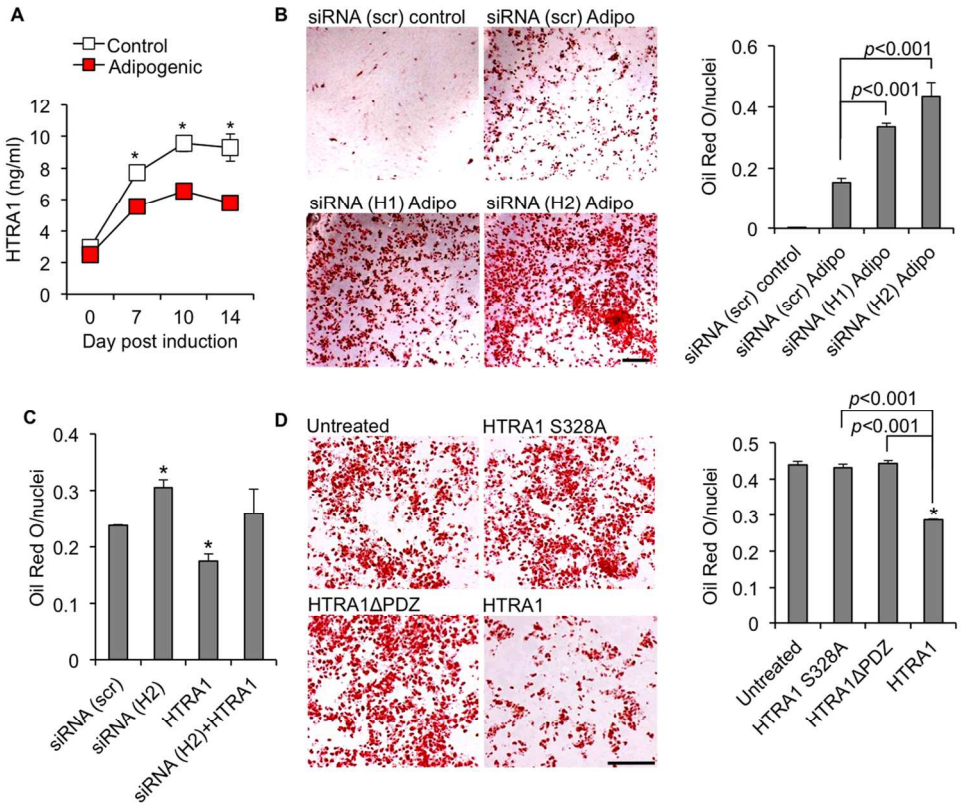

Figure 1. HTRA1 suppresses lipid uptake and droplet formation in hMSCs undergoing adipogenesis. **(A):** hMSCs were incubated in growth medium (control) or adipogenic induction medium (adipogenic) for up to 14 days and secreted HTRA1 protein levels determined by ELISA. *p < 0.001 as compared to hMSCs undergoing adipogenesis. **(B):** hMSCs were treated with scrambled siRNA (scr) or siRNAs specific for HTRA1 (H1 and H2) and induced to undergo adipogenesis. On day 14 following adipogenic induction, cells were stained using Oil Red O and oil droplet accrual quantified and normalized to cell number. Scale bar = 1 mm. **(C):** hMSCs were treated with siRNA specific for HTRA1 (H2), HTRA1 (45 nM) or a combination of both H2 and HTRA1 and induced to undergo adipogenesis. On day 14 following adipogenic induction, cells were stained using Oil Red O and oil droplet accrual quantified and normalized to cell number. *p < 0.01 as compared to siRNA (scr) treated cells. **(D):** hMSCs were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1), proteolytic inactive HTRA1 (HTRA1 S328A) or proteolytic active HTRA1 without PDZ domain (HTRA1ΔPDZ). On day 14 following adipogenic induction, cells were stained using Oil Red O and oil droplet accrual quantified and normalized to cell number. *p < 0.001 as compared to untreated. Scale bar = 1 mm. Data are representative of at least 2 separate experiments performed in triplicate. Abbreviations: hMSC, human mesenchymal stem cells; ELISA, enzyme-linked immunosorbent assay; HTRA1, high temperature requirement protease A1; siRNA, small interfering RNA.

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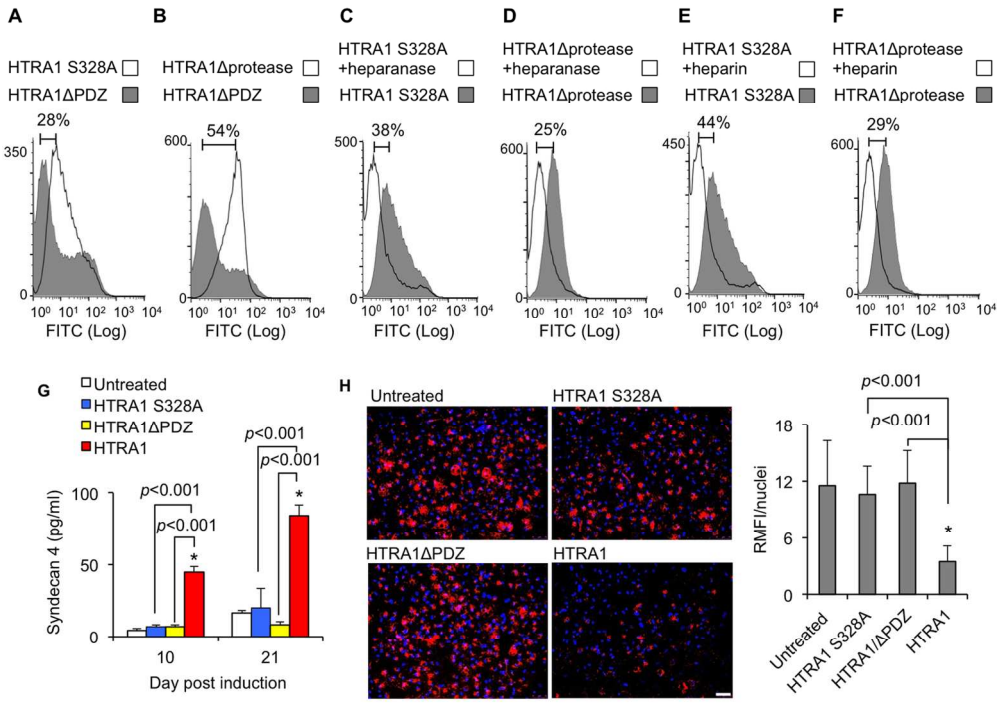

Figure 2. HTRA1 interacts with surface bound HSPG. **(A-F):** hMSC were incubated with recombinant his-labeled HTRA1 protein (0.45 µM) on ice for 1 h, washed and HTRA1 binding determined by FACS analysis using a FITC labeled anti-histidine antibody. hMSC binding of PDZ domain-containing HTRA1 S328A **(A)** or HTRA1Δprotease **(B)** as compared to HTRA1ΔPDZ. In order to investigate the involvement of HSPG in the binding of recombinant HTRA1 protein, cells were pre-treated with heparanase (10 U/ml) or heparin (10 µg/ml). hMSC binding of S328A **(C, E)** and HTRA1Δprotease **(D, F)** in the presence of heparanase **(C, D)** or heparin **(E, F)**. **(G):** hMSCs were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1), proteolytic inactive HTRA1 (HTRA1 S328A) or proteolytic active HTRA1 without PDZ domain (HTRA1ΔPDZ). ELISA measurement of soluble syndecan-4 were performed at day 10 and 21 post adipogenic induction. **(H):** hMSCs were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1), proteolytic inactive HTRA1 (HTRA1 S328A) or proteolytic active HTRA1 without PDZ domain (HTRA1ΔPDZ). At day 24 post adipogenic induction, hMSCs were rinsed once with PBS and incubated for an additional 3 h with DiI-labeled VLDL and carrier protein ApoE2. VLDL-DiI (red) uptake was quantified by measuring relative linear median fluorescence intensity (RMFI) using NIH ImageJ software and normalized to cell number. Nuclei were stained with DAPI (blue). Scale bar = 75 µm. *$p < 0.001$ as compared to untreated hMSCs. Data are representative of at least 2 separate experiments performed in triplicate. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1; HSPG, heparan sulfate proteoglycan; ELISA, enzyme-linked immunosorbent assay; FITC, Fluorescein isothiocyanate; VLDL-DiI, very low density lipoprotein-dialkylcarbocyanine.

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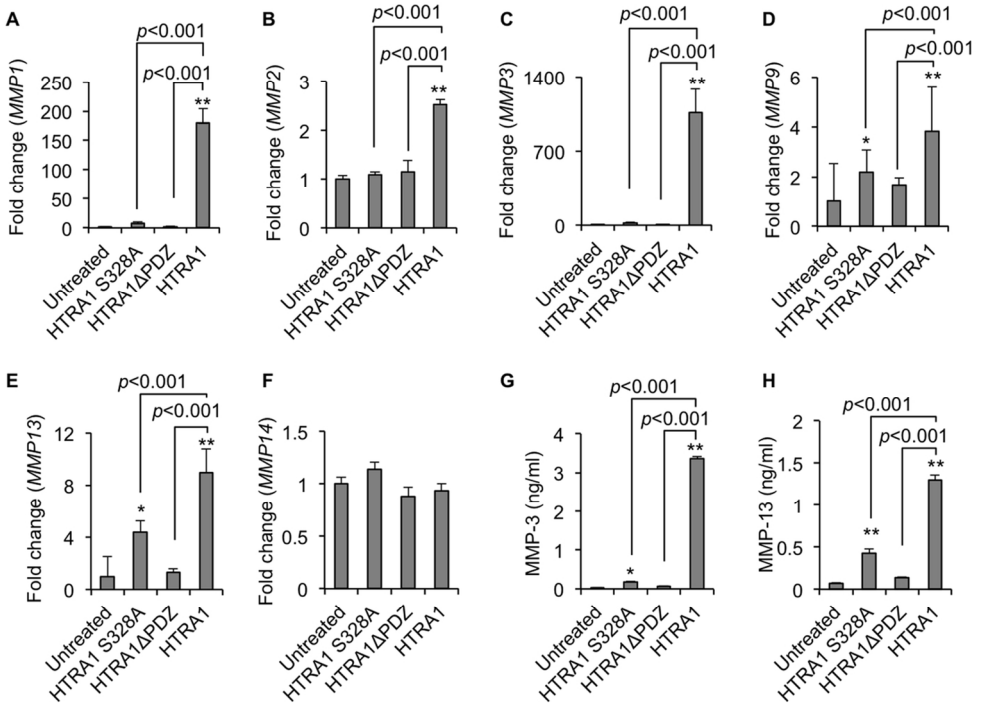

Figure 3. HTRA1 upregulates MMP production by hMSCs undergoing adipogenesis. hMSCs were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1), proteolytic inactive HTRA1 (HTRA1 S328A) or proteolytic active HTRA1 without PDZ domain (HTRA1ΔPDZ). At 21 days post adipogenic induction, expression levels of *MMP1* **(A)**, *MMP2* **(B)**, *MMP3* **(C)**, *MMP9* **(D)**, *MMP13* **(E)**, and *MMP14* **(F)** were determined by RT-qPCR. Data was normalized to *GUSB* and expressed as fold change as compared to untreated hMSCs (value = 1) using the comparative CT method. **(G, H):** hMSCs were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1), proteolytic inactive HTRA1 (HTRA1 S328A) or proteolytic active HTRA1 without PDZ domain (HTRA1ΔPDZ). At 21 days post adipogenic induction, levels of secreted MMP-3 **(G)** and -13 **(H)** were determined in hMSC supernatants using specific ELISAs. *$p < 0.05$, **$p < 0.001$ as compared to untreated hMSCs. Data are representative of at least 2 separate experiments performed in triplicate. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1; MMP, matrix metalloproteinase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

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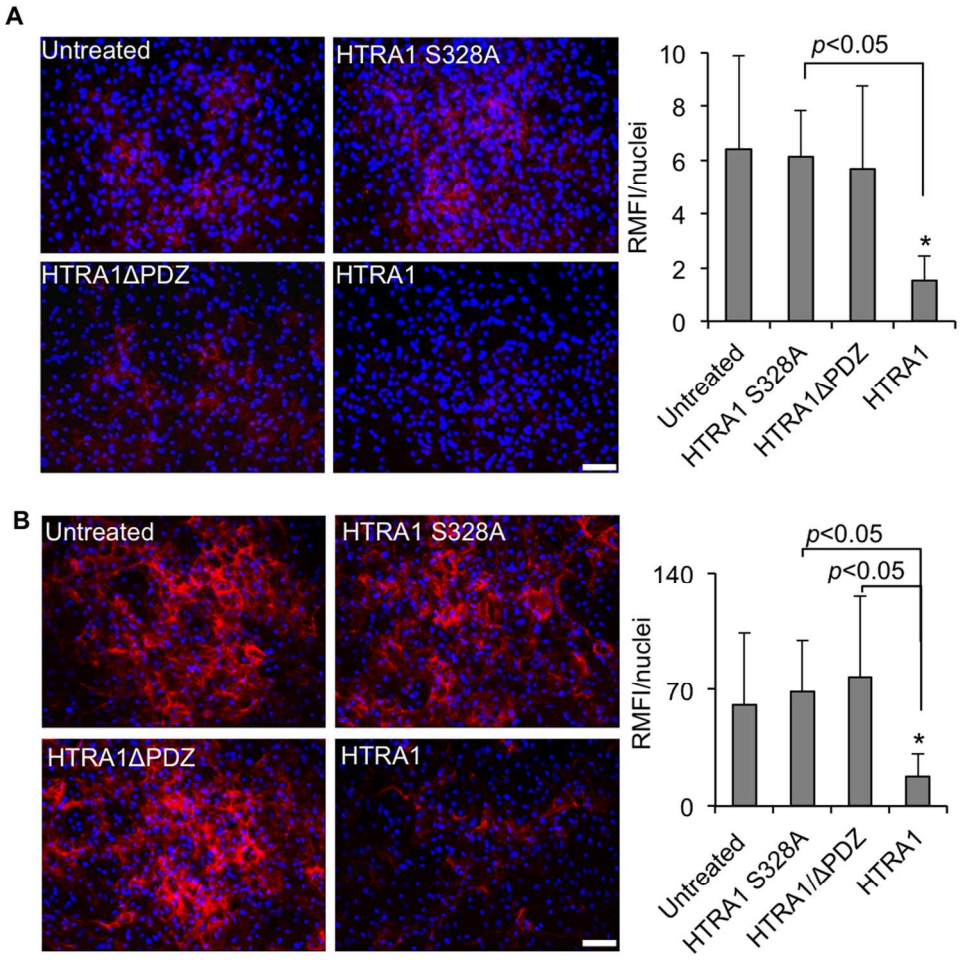

Figure 4. Immunofluorescence analysis of ECM. hMSCs were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1), proteolytic inactive HTRA1 (HTRA1 S328A) or proteolytic active HTRA1 without PDZ domain (HTRA1ΔPDZ). At day 24 post adipogenic induction, hMSCs were stained with antibodies specific for laminin **(A)** or type IV collagen **(B)** and positive staining detected using a Cy3-labelled secondary antibody (red). Immunofluorescence was quantified by measuring relative linear median fluorescence intensity (RMFI) using NIH ImageJ software and normalized to cell number. Nuclei were stained with DAPI (blue). Scale bar = 100 μm. *$p < 0.01$ as compared to untreated hMSCs. Data is representative of at least 2 separate experiments performed in triplicate. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1.
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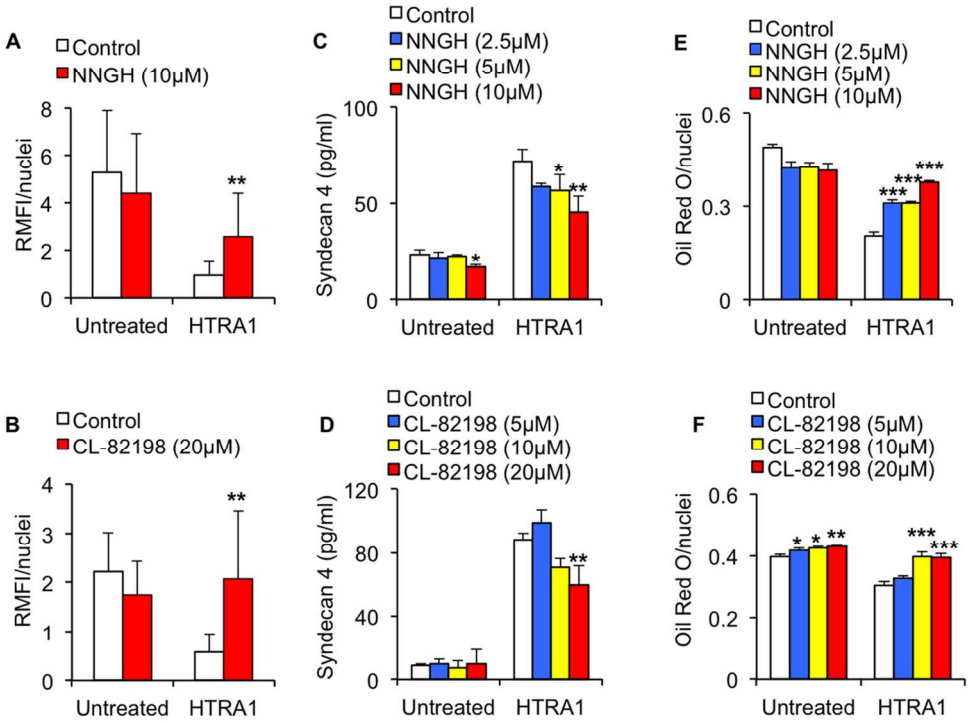

Figure 5. HTRA1's effects are mediated through MMP activities. **(A, B):** hMSCs pretreated with either vehicle control or with 10 μM of NNGH **(A)** or 20 μM of CL-82198 **(B)** were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1). At day 22 post adipogenic induction, hMSCs were stained with an antibody against type IV collagen and positive staining detected using a Cy3-labelled secondary antibody. Immunofluorescence was quantified by measuring relative linear median fluorescence intensity (RMFI) using NIH ImageJ software and normalized to cell number. **(C, D):** hMSCs pretreated with either vehicle control or with varying concentrations of NNGH **(C)** or CL-82198 **(D)** were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1). At day 17 post adipogenic induction, soluble syndecan-4 levels were measured in supernatants by specific ELISA. **(E, F):** hMSCs pretreated with either vehicle control or with varying concentrations of NNGH **(E)** or CL-82198 **(F)** were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1). At day 18 post adipogenic induction, cells were stained using Oil Red O and oil droplet accrual quantified and normalized to cell number. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared to controls from untreated or HTRA1-treated groups respectively. Data are representative of at least 2 separate experiments performed in triplicate. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1; MMP, matrix metalloproteinase; ELISA, enzyme-linked immunosorbent assay.
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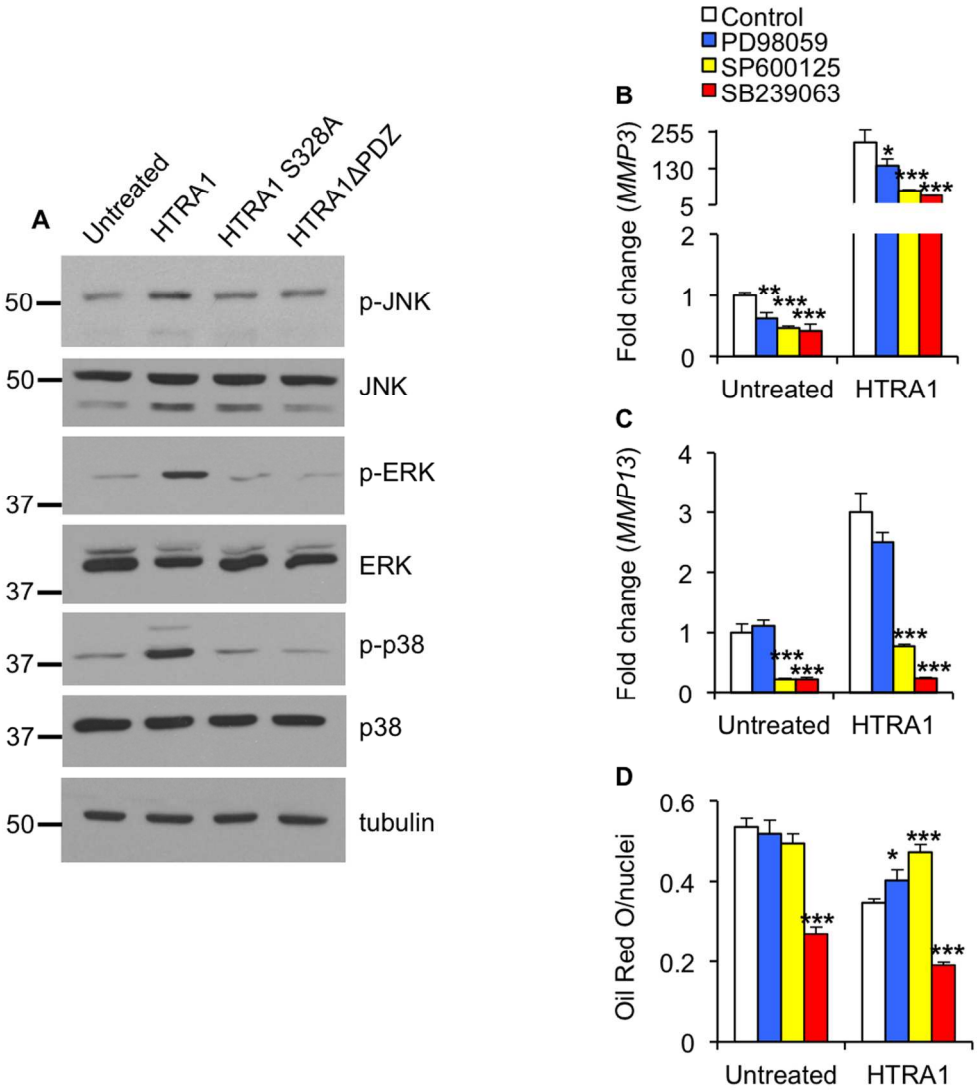

Figure 6. HTRA1's effects are mediated through MAP kinase activities. **(A):** hMSCs were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1), proteolytic inactive HTRA1 (HTRA1 S328A) or proteolytic active HTRA1 without PDZ domain (HTRA1ΔPDZ). At day 14 post adipogenic induction, protein lysates were subjected to Western blot analysis using antibodies specific for phosphorylated and non-phosphorylated JNK, p38 and ERK. An anti-tubulin monoclonal was used to confirm equal loading. **(B-D):** hMSCs pretreated with either vehicle control or with PD98059 (10 μM), SP600125 (20 μM) or SB239063 (10 μM) were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1). At day 17 post adipogenic induction, expression levels of *MMP3* **(B)** and *MMP13* **(C)** were determined by RT-qPCR. Data was normalized to *GUSB* and expressed as fold change relative to untreated, control hMSCs (value = 1) using the comparative CT method. **(D):** Oil Red O staining was also performed and oil droplet accrual quantified and normalized to cell number. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as compared to controls from untreated or HTRA1-treated groups respectively. Data are representative of at least 2 separate experiments performed in triplicate. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1; MMP, matrix metalloproteinase; MAP, mitogen-activated protein; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GUSB, Glucuronidase beta.

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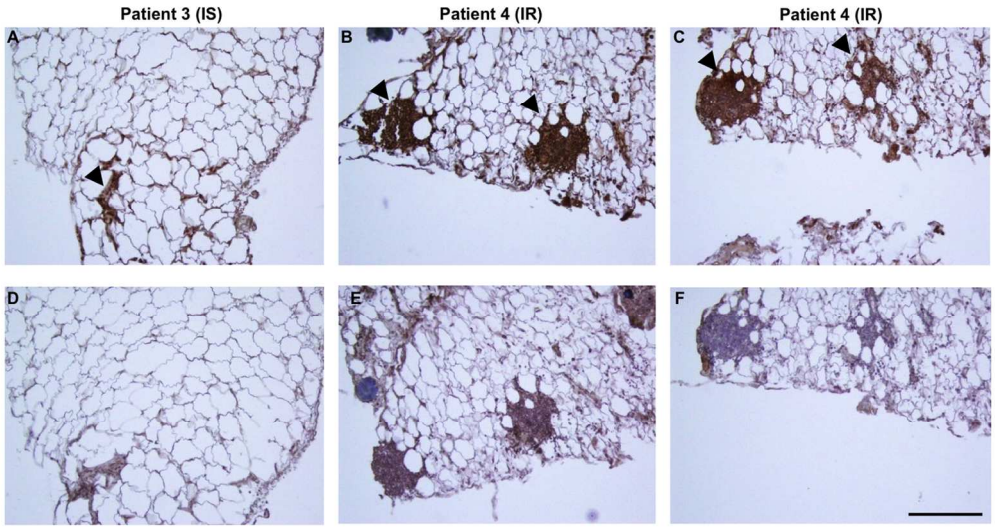

Figure 7. Identification of HTRA1 in adipose tissue. Paraffin wax sections of human visceral (omental) adipose tissue from insulin sensitive (IS) or insulin resistant (IR) obese patients were incubated with an anti-HTRA1 antibody **(A, B)**, anti-MMP-13 antibody **(C)** or relevant IgG controls **(D-F)**, and positive staining identified using an appropriate HRP-labeled polyclonal antibody with subsequent development using 3,3'-diaminobenzidine (brown). *Arrow heads*, HTRA1 or MMP-13 present in crown-like structures. Scale bar = 200 μm. Abbreviations: HTRA1, high temperature requirement protease A1; MMP, matrix metalloproteinase; HRP, horseradish peroxidase.
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Supplemental Table S1. List of Gene Expression Assays used in RT-qPCR analysis.

Gene	Protein Product	Assay ID [a]
HTRA1	High-Temperature Requirement Protease A1	Hs01016151_m1
MMP1	Matrix metalloproteinase 1 / Interstitial Collagenase	Hs00899658_m1
MMP2	Matrix metalloproteinase 2 / gelatinase A	Hs01548727_m1
MMP3	Matrix metalloproteinase 3 / Stromelysin-1	Hs00968305_m1
MMP9	Matrix metalloproteinase 9 / gelatinase B	Hs00234579_m1
MMP13	Matrix metalloproteinase 13 / Collagenase-3	Hs00233992_m1
MMP14	Matrix metalloproteinase 14 (membrane-inserted)	Hs01037003_g1
PPARG	Peroxisome proliferator-activated receptor gamma	Hs01115513_m1
FABP4	Fatty acid binding protein 4	Hs01086177_m1
CD36	Cluster of differentiation 36	Hs01567185_m1
ADIPOQ	Adiponectin	Hs00605917_m1
GUSB	Glucuronidase, beta	Hs99999908_m1

[a] TaqMan Expression Assay identity code according to supplier (Life Technologies, Zug, Switzerland).

Supplemental Table S2. Human patient information.

Patient	Sex	Age (yrs)	BMI (kg/m^3)	Insulin sensitivity group [a]
1	F	43	40.4	Insulin sensitive
2	F	42	49.1	Insulin resistant
3	M	43	50.7	Insulin sensitive
4	F	56	30.9	Insulin resistant

[a] Defined by the glucose infusion rate (GIR) during the steady state of an euglycemic hyperinsulinemic clamp as previously described [21].

Supplementary Fig. 1. (A): hMSCs were induced to undergo adipogenesis in the presence of varying concentrations of recombinant wild type HTRA1 (HTRA1) ranging from 0 to 45 nM, and its influence on oil droplet accrual determined by Oil Red O staining. *$p <$ 0.01 as compared hMSCs cultured in the absence of HTRA1. **(B):** hMSCs were incubated in growth medium (control), osteogenic induction medium (osteogenic) or adipogenic induction medium (adipogenic) for up to 14 days and endogenous HTRA1 protein levels measured in supernatants by specific ELISA. *$p < 0.001$ as compared hMSCs cultured in growth medium at the equivalent time points. **(C):** hMSCs were treated with scrambled siRNA (scr) or siRNAs specific for HTRA1 (H1 and H2) and induced to undergo adipogenesis. Endogenous levels of HTRA1 were measured in supernatants at day 3 post adipogenic induction using a specific ELISA. *$p < 0.001$ as compared to scrambled siRNA (scr) treated cells. **(D):** Schematic of recombinant human HTRA1 proteins; active HTRA1 (ΔMac), inactive HTRA1 (HTRA1 S328A), active HTRA1 without PDZ domain (HTRA1ΔPDZ), inactive HTRA1 without PDZ domain (HTRA1 S328AΔPDZ) and HTRA1 without protease domain (HTRA1Δprotease). The purified recombinant histidine-labeled human HTRA1 proteins were also visualized on a Coomassie blue-stained SDS-PAGE gel. *M*, protein marker; *lane 1*, HTRA1; *lane 2*, HTRA1 S328A; *lane 3*, HTRA1ΔPDZ; *lane 4*, HTRA1 S328AΔPDZ; *lane 5*, HTRA1Δprotease. **(E):** Proteolytic activity of recombinant HTRA1 proteins (45 nM) towards BODIPY-FL-labeled DQ elastin (25 μg/ml) at 37^{o}C as determined using a Multiplate reader. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1; ELISA, enzyme-linked immunosorbent assay; siRNA, small interfering RNA; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Supplementary Fig. 2. Influence of HTRA1 on adipogenic markers. hMSCs were induced to undergo adipogenesis in the absence (untreated) or presence of 45 nM of recombinant wild type HTRA1 (HTRA1), proteolytic inactive HTRA1 (HTRA1 S328A) or proteolytic active HTRA1 without PDZ domain (HTRA1ΔPDZ). At 21 days post adipogenic induction, expression levels of *PPARG* **(A)**, *FABP4* **(B)**, *CD36* **(C)** and *ADIPOQ* **(D)** were determined by RT-qPCR. Data was normalized to *GUSB* and expressed as fold change as compared to untreated hMSCs (value = 1) using the comparative C_T method. *$p < 0.01$ as compared to untreated cells. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1; PPARG, peroxisome proliferator-activated receptor gamma; FABP4, fatty acid binding protein 4; CD36, cluster of differentiation 36; GUSB, Glucuronidase beta; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Supplementary Fig. 3. FACS analysis of recombinant HTRA1 binding to non-differentiated hMSCs. HTRA1Δprotease (0.45 μM) was incubated with hMSCs for 1 h at 4^{o}C and cell-bound HTRA1 protein detected using a FITC labeled anti-his antibody. The influence of pre-incubating cells with collagenase (1 mg/ml) **(A)**, chondroitinase (10 U/ml) **(B)**, CS1 (50 μM) (**C**) or GRGDSP (50 μM) **(D)** on the percentage of cell-bound HTRA1Δprotease was calculated using FlowJo 10 software. Data are representative of least two individual experiments performed in duplicate. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1; FACS, fluorescence activated cells sorting.

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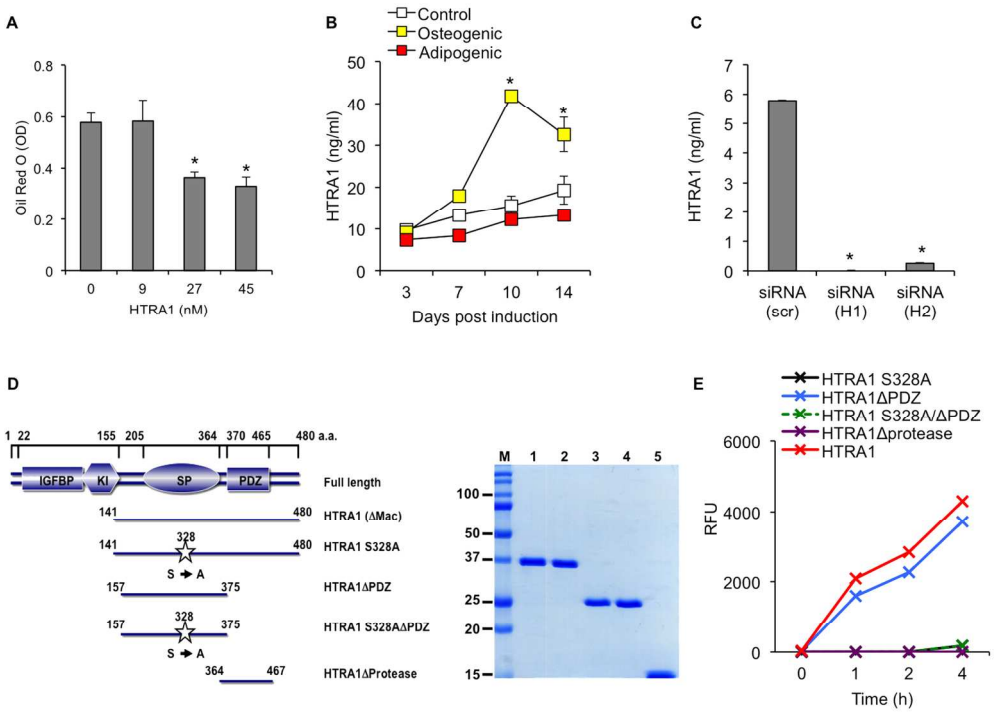
or with CL-82198 (20 μM), HTRA1 (45 nM) or HTRA1 and CL-82198. Cell supernatants were harvested, concentrated and subjected to Western blot analysis using an antibody specific for the amino-terminal fibrin- and heparin-binding domain of fibronectin. *p < 0.05, **p < 0.01, ***p < 0.001 as compared to siRNA (scr) controls from untreated or HTRA1-treated groups respectively. Data are representative of least two individual experiments performed in triplicate. *Arrow heads* indicate the 29 kDa Fnf due to HTRA1 treatment. **(D):** Coomassie stained gel of total protein from supernatants to control for equal loading. Data are representative of least two individual experiments. Abbreviations: hMSC, human mesenchymal stem cells; HTRA1, high temperature requirement protease A1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GUSB, Glucuronidase beta; MMP, matrix metalloproteinase; siRNA, small interfering RNA; Fnf, fibronectin fragments.

Supplementary Fig. 7. Identification of HTRA1 in adipose tissue. Paraffin wax sections of human visceral (omental) adipose tissue from IS or IR obese patients were incubated with an anti-HTRA1 antibody **(A, B)** or relevant IgG controls **(C, D)**, and positive staining identified using an appropriate HRP-labeled polyclonal antibody with subsequent development using 3,3'-diaminobenzidine (*brown*). *Asterisks*, HTRA1-positive blood vessels. Scale bar = 200 μm. Insulin sensitivity was defined as glucose infusion rate (GIR) during the steady state of an euglycemic-hyperinsulinemic clamp > 70 μmol/kg/min, insulin resistance as GIR < 50 μmol/kg/min. Abbreviations: HTRA1, high temperature requirement protease A1; IS, insulin sensitive; IR, insulin resistant; HRP, horseradish peroxidase.

Supplementary Fig. 8. High power image of HTRA1 in human visceral (omental) adipose tissue from obese patient 4 (IR). Paraffin sections were incubated with an anti-HTRA1 antibody and positive staining identified using an appropriate HRP-labeled polyclonal antibody with subsequent development using 3,3'-diaminobenzidine (*brown*). Scale bar = 20 μm. Abbreviations: HTRA1, high temperature requirement protease A1; IR, insulin resistant; HRP, horseradish peroxidase.



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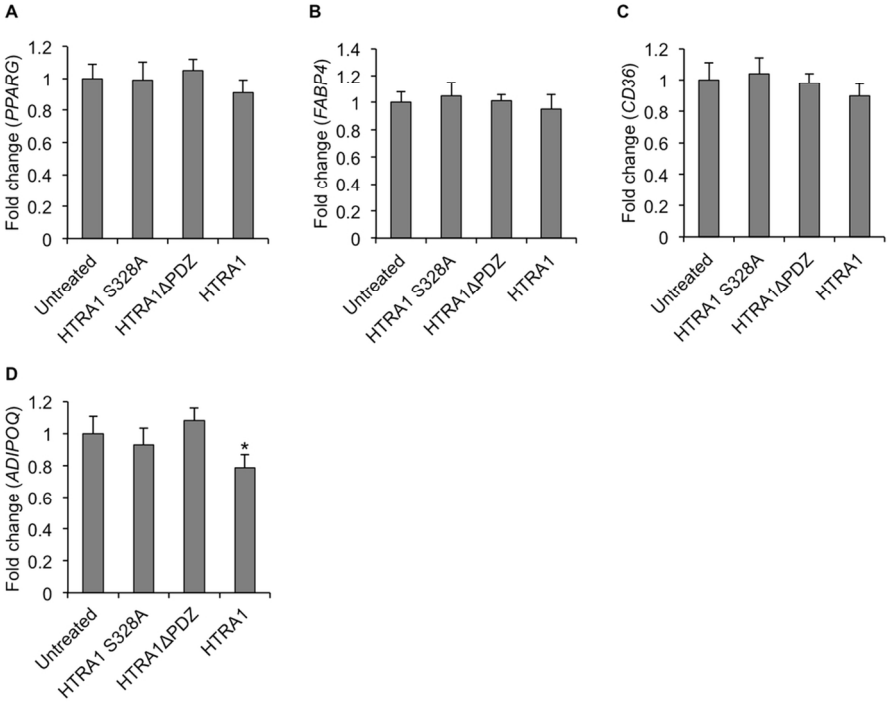

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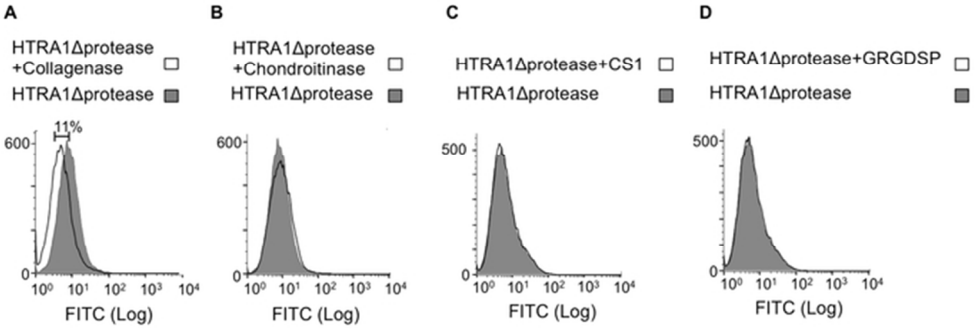

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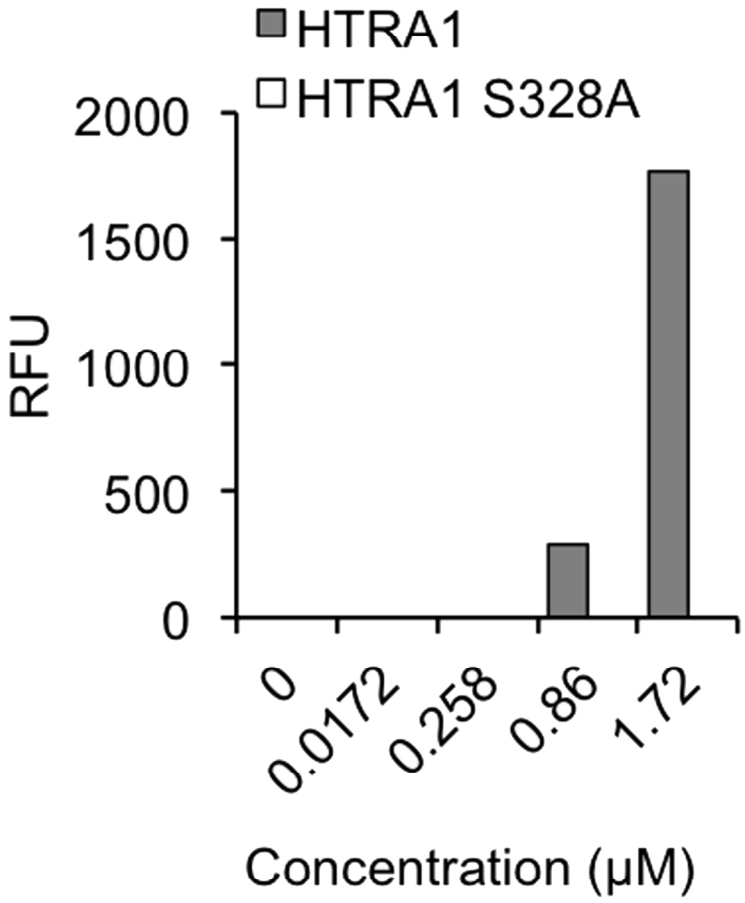

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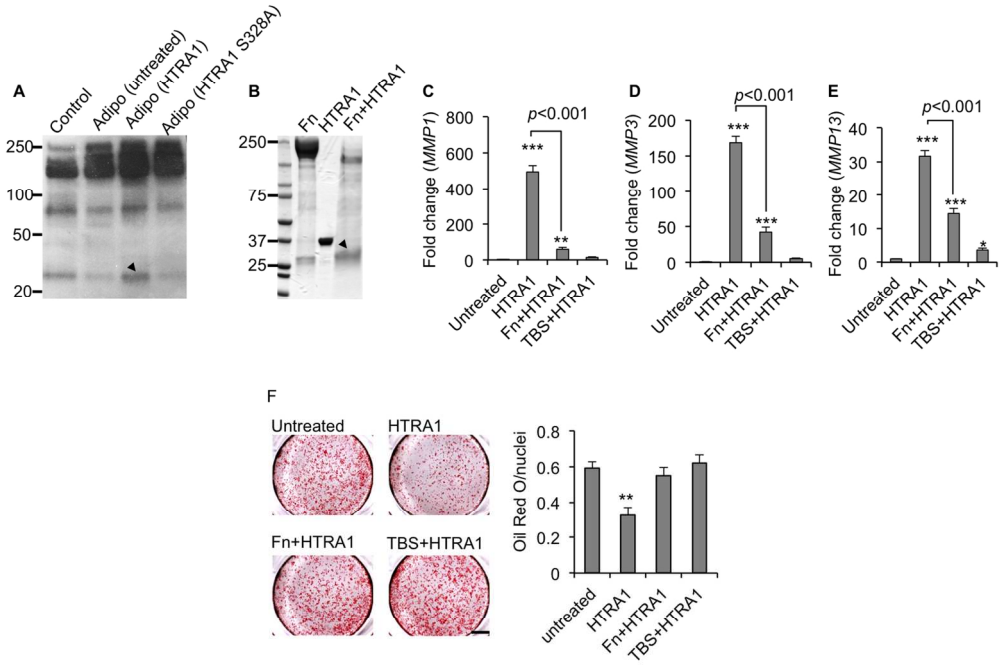

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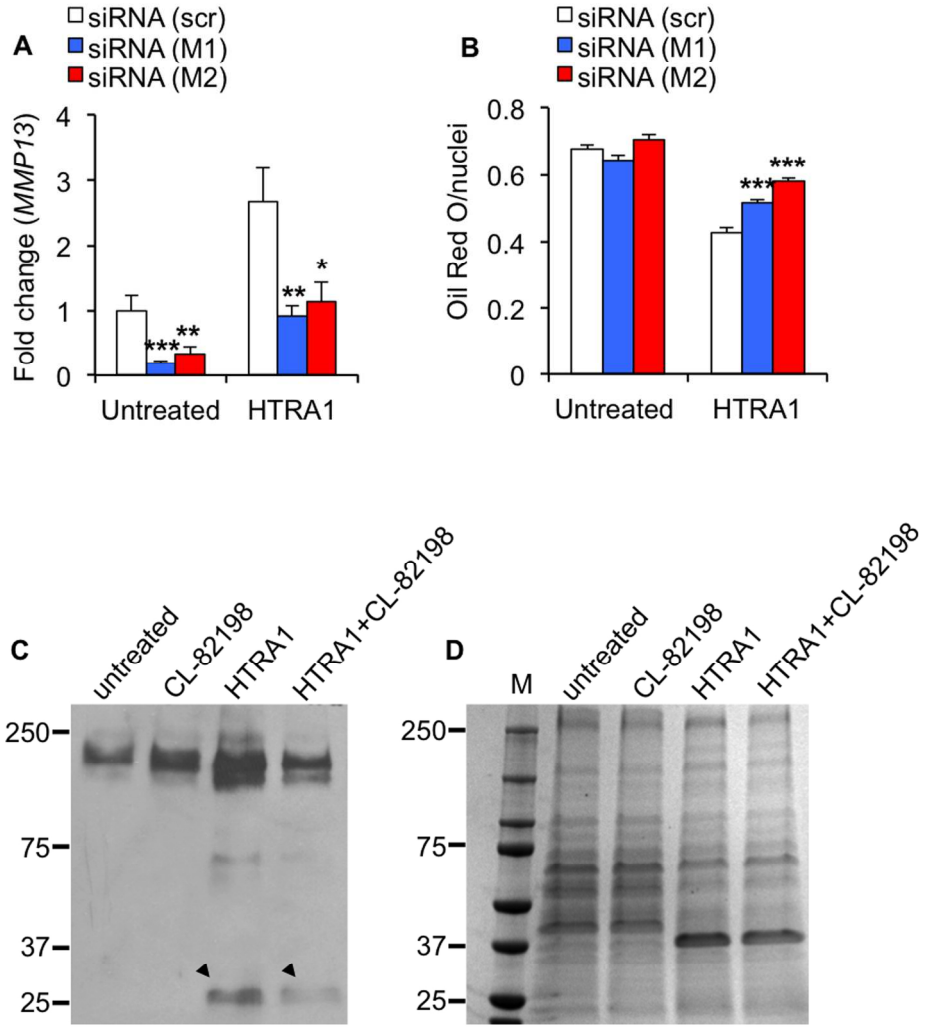

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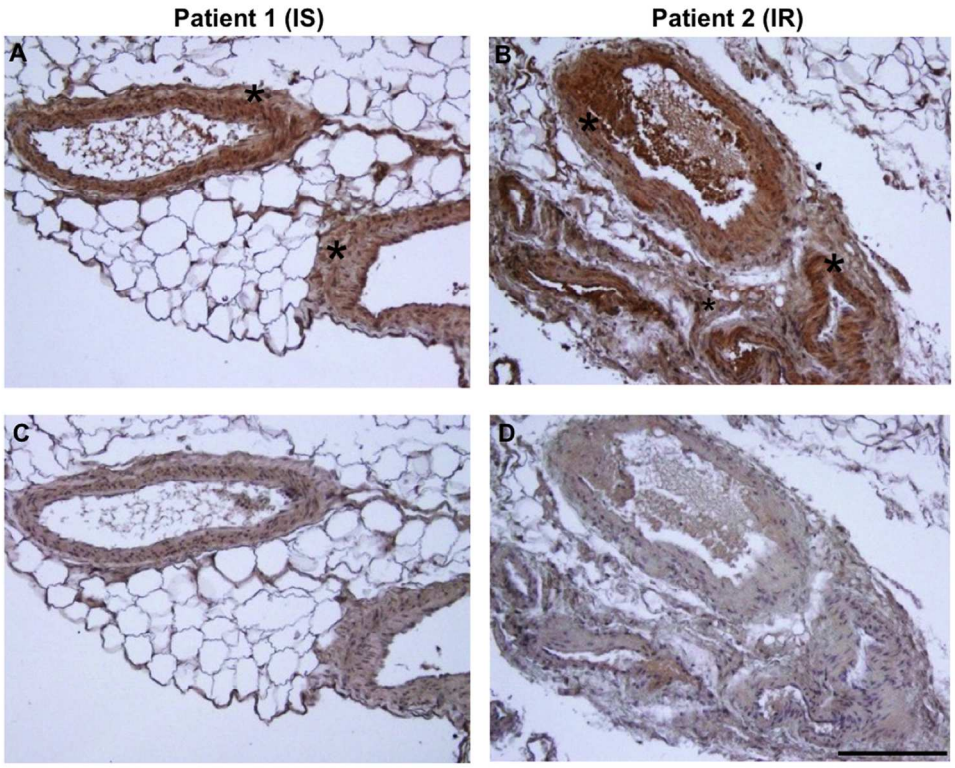

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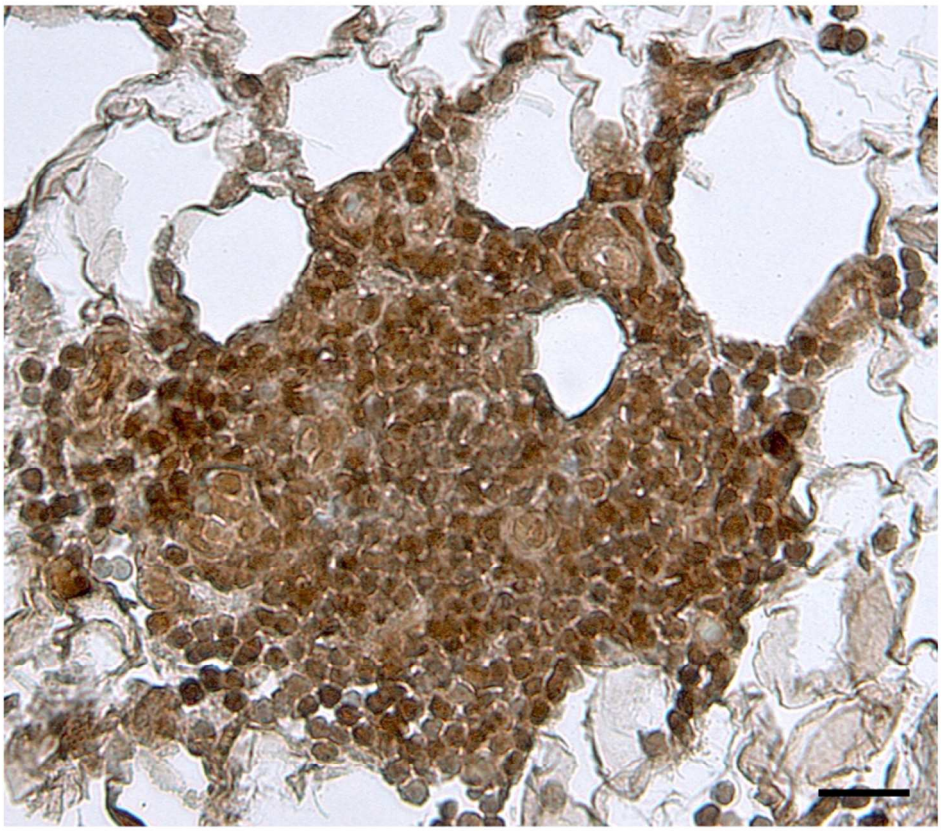

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